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Enhanced Retrieval of DNA from Human Fecal Samples Results in Improved Performance of Colorectal Cancer Screening Test

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Colorectal cancer accounts for more than 10% of all cancer deaths but is curable, if detected early. We reported previously on a stool-based screening test in which DNA from stool samples is subjected to genome analysis; sensitivity of the test has been limited in part by inefficiency of retrieving DNA from stool. Our aim was to test the impact of a new purification method that would increase the yield of human DNA from stool. DNA from 86 cancer and 100 non-cancer subjects (diagnosed by colonoscopy) were purified from stool with a new method for DNA recovery based on sequence-specific capture with acrylamide gel immobilized capture probes as well as with a previously developed magnetic bead-capture procedure. The new purification method gives an average 5.4-fold increase in the quantity of human DNA that can routinely be retrieved from fecal samples. The increased recovery of DNA corresponds with an increase in assay sensitivity from 53% (CI: 42 to 64%) to 70% (CI: 59 to 79%); $P = 0.0005$ (by McNemar's test), with no change in specificity. The newly developed sample preparation method mitigates a major problem in detecting rare cancer-associated genetic changes in heterogeneous clinical samples such as stool. ***(J Mol Diagn 2004, 6:386–395)***

Colorectal cancer (CRC) is curable in more than 90% of cases when caught in the earliest stages. Current colorectal cancer screening guidelines include a variety of options. Colonoscopy may be the most sensitive screening test,[1] however its invasiveness (including bowel preparation and the procedure itself) present major barriers to its implementation for large-scale, nationwide screening.[2] An improved non-invasive screening option could address many of the issues associated with colonoscopy. Non-invasive screening is available today through assessment of occult blood in fecal samples, but this test has relatively low sensitivity, especially for early stage cancer, limiting its impact on cancer mortality. However, analysis of DNA from stool provides an attractive, alternative, non-invasive means for CRC screening if scalable, sensitive, and specific tests can be developed.

We have previously described[3] a stool-based screening test for early detection of colorectal cancers. The multi-target nucleic acid assay consists of a panel of 21 specific mutations in adenomatous polyposis coli (*APC*),[4] *p53* [5,6], and K-*ras*[7] genes, a microsatellite instability marker (BAT-26),[8] and a marker for genomic integrity (DNA Integrity assay; DIA).[9] As reported in separate studies, [3,10,11,12] the multi-target assay has an aggregate sensitivity of 67% (95% CI: 60.3 to 73.9%) and specificity of 97% (95% CI: 92.9 to 99.2%), a major improvement to the current screening methods of the fecal occult blood test (25 to 40% sensitivity).[13,14] In the multi-target assay studies human DNA was recovered and purified using streptavidin-bound magnetic beads.[3,15] We have reported on the use of separate components of this multi-target test elsewhere.[9, 11,16,17,18] The mutation panel portion of the multi-target assay relies on detecting mutations in several well-documented colorectal cancer-associated genes.[19,20] The DNA integrity portion of the test consists of a set of markers that serve as surrogate markers for the presence of long DNA fragments. The principles and performance of this portion of the test has recently been reported.[9]

During development of the assay we observed that the sensitivity of a gene or genome-based test is limited not only by the fact that not all colorectal tumors have identified mutations, but also by the quantity of tumor-derived DNA that can be retrieved from stool. Robust and reproducible recovery of sufficient target DNA from stool is often

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an unrecognized, but significant challenge for developing a population-based screening assay. The content of human DNA in stool is very small, although the total DNA that can be recovered is very high due to bacterial contribution. DNA from cells sloughed from the colonic mucosa represents as little as 0.1 to 0.01% of the total DNA recoverable from stool. Additionally, the human DNA is highly heterogenous. Tumor cells in the colon can be estimated to contribute on the order of 1% of the cells sloughed, although the amount can vary, and with early stage disease the mutant percentage can be less than 1%.[21,22]

To maximize sensitivity of detecting mutant DNA for a screen of CRC in an asymptomatic population it is important to maximize the recovery of target DNA from stool. Insufficient recovery would lead to the possible absence of mutant molecules within PCR reactions, leading to false negative results, and reduced clinical sensitivity. We introduce here a novel DNA purification technology that consists of an electrophoretic driven separation of target DNA sequences, using oligonucleotide capture probes immobilized in an acrylamide gel. The amount of sample to be purified can be easily scaled to recover increasing quantities of target DNA from stool, using this approach. Using the sequence-specific electrophoretic capture method, we have demonstrated that adequate genome representation in the sample is a limiting factor for DNA-based detection of colorectal cancer and maximizing representativeness through increased recovery improves clinical sensitivity. The population of mutant sequences in the human DNA recovered from stool can be modeled by Poisson statistics. Based on a nominal 1% mutant in the human DNA, it is predicted that a minimum of 500 copies are required for high probability (99%) of detection. Likewise, for early stage disease, where the mutant population may represent less than 1% of the total human DNA recovered from stool, the minimum copies required for robust detection increases (eg, 2500 copies for 0.2% mutant DNA).

In this study a total of 186 archived stool samples were analyzed using the multi-target assay, after recovery of DNA using two different purification techniques; magnetic bead-capture and the gel-capture method. In development experiments the gel-capture approach was shown to yield increased recovery of human DNA from stool, due primarily to the ability to load more sample without overloading the sequence-specific capture layer. This study included a set of 86 archived samples from cancer patients, which had been previously analyzed.[10–12] The impact of increased DNA recovery was expected to increase the detection of mutations, and clinical sensitivity, due to maximized representation of mutant sequences in PCR reactions.

Materials and Methods

Sample Collection and Preparation

All stool samples ($N = 186$) were frozen within 24 to 72 hours after collection, and stored at −80°C. For recovery of human DNA, samples were thawed at room temperature and homogenized in an excess volume (1:7, wt:vol) of EXACT buffer A (EXACT Sciences, Marlborough, MA) using an EXACTOR stool shaker (EXACT Sciences). After homogenization, a 4-g stool equivalent of each sample was centrifuged to remove all particulate matter. The supernatants were then treated with 20 μl TE buffer (Pierce, Rockford, IL) (0.01 mol/L Tris [pH 7.4] and 0.001 mol/L EDTA) containing RNase A (Roche, Indianapolis, IN) (2.5 mg/ml), and incubated at 37°C for 1 hour. Total nucleic acid was then precipitated (first adding 1/10 volume 3 mol/L NaAc (Sigma, St. Louis, MO), then an equal-volume of isopropanol). Genomic DNA was pelleted by centrifugation, the supernatant removed, and the DNA resuspended in TE. For magnetic bead-based purification, the volume of TE buffer added was 10 ml; for the acrylamide gel-based purification, the volume of TE added was 4 ml. For each group of samples prepared, process positive-control samples as well as component negative controls were included.

Archived samples were stored at −80°C for an average of 12 months (range of 6 to 18 months) for use in this study. Integrity of recovered DNA was stable under these storage conditions as indicated by repeat analysis of samples.

Magnetic Bead-Based Sequence-Specific Purification[15]

Sequence-specific DNA fragments were purified from the total nucleic acid preparations by performing oligonucleotide-based hybrid captures. For each sample, seven unique hybrid capture reactions were performed in duplicate. Each capture reaction was carried out by adding 300 μl of sample preparation to an equal volume of 6 mol/L guanidine isothiocyanate solution (GITC), (GIBCO, Invitrogen, Carlsbad, CA) containing biotinylated sequence-specific oligonucleotides (20 pmol; Midland Certified Reagent Co., Midland, TX). The mixture was heated to 95°C, then rapidly cooled to room temperature, and after a 2-hour incubation at 25°C, the GITC was diluted to 1 mol/L concentration. Streptavidin-coated magnetic beads (Dynal, Oslo, Norway) were added to the solution, and the tubes were incubated for an additional hour at room temperature. The bead/hybrid capture complexes were then washed 4 times with 1X B&W buffer (Dynal), (1 mol/L NaCl, 0.01 mol/L Tris-HCl [pH 7.2], 0.001 mol/L EDTA, and 0.1% Tween 20), and the sequence-specific captured DNA was eluted into 35 μl TE by heat denaturation.

Acrylamide-Gel Method for DNA Purification

Target human DNA fragments were purified from total nucleic acid preparations by electrophoretically driving DNA through an affinity capture layer consisting of human, sequence-specific capture probes immobilized within an acrylamide matrix. Capture probes were synthesized as 37-mer oligonucleotides with a 5′-Acrydite[23,24] modifications (Integrated DNA Technologies, Coralville, IA). The capture probes were prepared as 1

mmol/L stock solutions in 0.1X TE buffer. The polymerization solution (1 ml total) was then prepared by mixing 119 μl acrylamide:bisacrylamide (19:1) (Roche), 20 μl of each Acrydite capture probe, 100 μl 10X Tris Borate EDTA (TBE) buffer (BioRad, Hercules, CA), 20 μl glycerol (OmniPur, Darmstadt, Germany), 22 μl dimethylformamide (Sigma), 668 μl MB-grade water (Sigma), 10 μl freshly prepared 10% Ammonium Persulfate (APS) (Sigma), and 1 μl Tetramethylethylenediamine (TEMED) (Sigma). This formulation resulted in a 5% polyacrylamide gel and 20-μmol/L concentration of capture probes. Unique mixtures were prepared for the mutation panel (consisting of a multiplexed capture of 11 unique sequences), and each of the four DIA sequences. A sheet of medical-grade polyester (SEFAR, Depew, NY), with 100-um openings, was treated with 0.5% SDS (from 10% stock, GIBCO) and dried. The sheet was then clamped between glass plates, and the polymerization mix was wicked into the sheet and allowed to polymerize for several hours. The glass plates were then separated, the gel allowed to dry, and 1-cm diameter disks were cut out. The disks were heat sealed to the bottom of a custom-molded polypropylene capture plate, consisting of 48 wells, each approximately 1 cm in diameter, and 1.5 cm in height. A matched array of 48 molded tubes was then fitted into the wells of the capture plate to accommodate up to 4 ml of sample per capture disk.

Crude human DNA preparations (2400 μl) were mixed with 960 μl formamide (Sigma), 385 μl 10X TBE, and filtered through a 0.8-um syringe filter (Nalgene, Rochester, NY), then denatured (heated at 95°C for 10 minutes, then cooled in ice for 5 minutes). First, 600 μl of 0.5% Seakem LE agarose (Cambrex, Rockland, ME) was added on top of the bonded capture membrane, and allowed to gel. The sample mix was then loaded on top of the agarose, and electrodes above and below the capture layer were applied. Samples were electrophoresed (15V, 16 hours) using TBE in the reservoirs above and below the capture layer. After electrophoretic capture the remaining solution was removed from the tubes, and the tube array (containing the agarose layer) was separated from the capture plate. The capture membranes were then washed with ST buffer (Sigma) (0.15 mol/L NaCl + 10 mmol/L Tris; pH 7.4) and the capture membranes were electrophoresed in the reverse direction (30V, 3 hours), and rinsed with ST buffer. Capture membranes were found to have sufficient porosity that captured DNA could be efficiently recovered simply using centrifugation. Therefore, 40 μl of 100 mmol/L NaOH (Sigma) was added to the top of the capture membrane and incubated for 15 minutes. The capture plate was placed on top of a custom molded 48-well DNA collection plate and centrifuged briefly (1900 × *g*) to recover the eluted DNA. Then, 8 μl of neutralization buffer (Sigma) (500 mmol/L HCL + 0.1X TE) was added to each well of the collection plate and mixed.

Sequence-Specific Amplification

Polymerase chain reaction (PCR) amplifications (50 μl) were performed on MJ Research Tetrad Cyclers (MJ Research, Watertown, MA) using 10 μl of purified DNA, 10X PCR buffer (Takara Bio Inc; Madison, WI), 0.2 mmol/L dNTPs (Promega, Madison, WI), 0.5 μmol/L sequence-specific primers (Midland Certified Reagent Co.), and 2.5 U LATaq DNA polymerase (Takara). All amplification reactions were performed under identical thermocycler conditions. After an initial denaturation of 94°C for 5 minutes, PCR amplification was performed for 40 cycles consisting of 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C, with a final extension of 5 minutes at 72°C. Thirteen separate PCR reactions were run per sample. For analysis of each of the PCR products, 8 μl of each amplification reaction was loaded and electrophoresed on a 4% ethidium bromide-stained NuSieve 3:1 agarose gel (Cambrex) and visualized with a Stratagene EagleEye II (Stratagene, La Jolla, CA) still image system. All oligonucleotide sequences [capture probes, PCR primers, and TaqMan probes] are available on request.

The multi-target assay was designed to have 13 separate PCR reactions in the multiple mutation (MuMu) panel, and 16 PCR reactions in the DIA portion of the assay. Two of the PCR reactions are overlapping for MuMu and DIA.

Mutation Panel Analysis

The presence or absence of point mutations or Bat-26-associated deletions was determined by using modified solid-phase single-base extension (SBE) reactions. Point mutation targets included; codons K12p1, K12p2, and K13p2 on the K-*ras* gene; codons 876, 1306, 1309, 1312, 1367p1, 1378p1, 1379, 1450p1, 1465, and 1554 on the *APC* gene; and codons 175p2, 245p1, 245p2, 248p1, 248p2, 273p1, 273p2, and 282p1 on the *p53* gene. Including the Bat-26 deletion marker, the panel consisted of 22 markers in total. For all gene targets, separate wild-type and mutant-specific reactions were performed. The following procedure was used: PCR product (42 μl) was added to 200 μg magnetic beads (Dynal) in 40 μl of 2X B&W buffer (Dynal), and incubated at room temperature for 15 minutes. Beads were then magnetically separated, the supernatant removed, and fresh B&W buffer was added and mixed. This process was repeated twice to wash the beads, then 100 μl of 0.1N NaOH was added to dissociate the bound double-stranded PCR product. The beads were then washed with B&W buffer, and the beads were finally placed in 100 μl TE. Wild-type reactions were run with fluorescently labeled nucleotides complementary to the wild-type base added. For each of the point mutation-specific reactions, fluorescently labeled bases complementary to the expected mutant bases were added in addition to unlabeled dideoxy nucleotides complementary to the wild-type base. Specific mutant reaction mixes varied from site to site and were dependent on the expected base at the mutation site of interest (in some cases more than a single mutation is possible). All SBE reactions are 10-μl total volume. Mutant-specific reactions are prepared using 5 μl bead-bound PCR template, 1 μl 10X buffer (Perkin Elmer, Boston,

Table 1. Summary of Cancer Detection Sensitivities from Previous Studies Utilizing Magnetic Bead Capture for DNA Preparations from Stool Samples

Study	Cancer samples detected	Sensitivity	Normal samples detected	Specificity
Ahlquist [3]	20/22	91%	2/28	92.9%
Brand[10]	11/16	69%	n/a	
Tagore[11]	33/52	63%	2/113	98.2%
Syngal[12]	40/65	62%	n/a	
Data on file	28/41	68%	n/a	
Overall	132/196	67.3% (95% CI: 60.3–73.9%)	4/141	97.2% (95% CI: 92.9–99.2%)

MA), 1 μl SBE primer (5 μmol/L), 0.025 μl AcycloPol enzyme (32 Units/μl) (Perkin Elmer), and a mixture of unlabeled dideoxynucleotides (Promega) and R110-labeled Acycloterminators (Perkin Elmer), dependent on the specific mutant site. Acycloterminators are diluted 1:20 from the stock solution as purchased; 0.05 μl of the diluted reagent is used per mutant base. Dideoxynucleotides are first prepared as a 50-μmol/L stock solution and then 1 μl of the stock solution is added to reactions. As an example, for k12p1 (where the wild-type sequence calls for G, but A, C, and T mutations are all possible), 1 μl of the ddGTP, and 0.05 μl of the R110-A, R110-C, and R110-T Acycloterminators are added to the reaction mix). Bat-26 mutations associated with a deletion of 4 to 15 bp (bp) were identified by size discrimination of reaction products.

All samples were analyzed on an ABI 3100 capillary electrophoresis (CE) system (ABI; Foster City, CA). Labeled primer extension products were prepared for analysis on the CE, as follows. An aliquot (1 μl) of primer extension product was mixed with 9 μl of a pre-mixed formamide/ROX standard solution (190 to 6 μl, respectively). The ROX mix, which serves as a size standard, consists of 5 ROX-labeled oligonucleotides of lengths 15, 18, 25, 30, and 50 bases, dissolved in 10 mmol/L Tris-EDTA buffer. Just before analysis, mixed samples were denatured on a thermocycler at 95°C for 5 minutes, then cooled on ice for 5 minutes. Samples were analyzed on the CE using 36-cm capillary arrays (ABI) and POP-6 (ABI) in the capillaries. Run temperature was set to 60°C, the operating potential set to 15V, and samples were electrokinetically injected at 3V. Data were analyzed using the GenoTyper software package.

DNA Integrity Assay (DIA)

The DIA assay has been previously described in detail.[9] More recently this assay has been converted to a real-time PCR methodology. Three unique PCR reactions (in duplicate) per loci were run on I-Cycler instruments (Bio-Rad). The strategy was to capture locus-specific segments and perform small (~100 bp) PCR amplifications remote from the capture site as an indicator of DNA length. DNA fragments for integrity analysis were amplified from four different loci: 17p13; 5q21; HRMT1L1; LOC91199. PCR primer sets and associated TaqMan probe for each loci of interest are "walked" down the chromosome thereby interrogating for the presence and quantitation of increasing length DNA of approximately 100-bp, 1300-bp, 1800-bp, and 2400-bp fragments of captured DNA. Purified DNA template (5 μl) was mixed with 5 μl 10X PCR buffer (Takara), 10 μl dNTP's (2 mmol/L) (Promega), 0.25 μl LATaq (5 U/μl; Takara), 24.75 μl molecular biology-grade water (Sigma), 5 μl of a mix of PCR primers (5 μmol/L; Midland) and TaqMan dual-labeled probes (2 μmol/L; Biosearch Technologies, Novato, CA). The I-Cycler was programmed as follows: 94°C for 5 minutes, then 40 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. Genomic standards, prepared as 20, 100, 500, 2500, and 12500 GE/5 μl were prepared and used to generate a standard curve.

DIA Data Analysis

Threshold genome equivalents (GE) values were determined for each of 12 PCR reactions (corresponding to the 1.3-kb, 1.8-kb, and 2.4-kb fragments across the four genomic loci) using a previously determined set of cancers and normals. We then applied a requirement that at least 4 of the 12 PCR reactions are above the individual PCR thresholds to prospectively determine cancers.

Quantification of Recovered DNA by TaqMan Analysis

TaqMan analysis was performed on an I-Cycler with primers against a 200-bp region of the *APC* gene. A probe labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) was used to detect PCR product. Amplification reactions consisted of captured human stool DNA mixed with 10X PCR buffer, LATaq enzyme (Takara), 1X PCR primers (5 μmol/L), and 1X TaqMan probe (2 μmol/L; Biosearch Technologies). We used 5 μl of captured DNA in the PCR reactions. TaqMan reactions were performed with the same program as described above (DIA).

Results

Sensitivity of Detection of Colorectal Cancer by a Multiple Genetic Target Analysis

In previous studies,[3,10–12] human DNA was recovered from homogenized stool samples collected from cancer

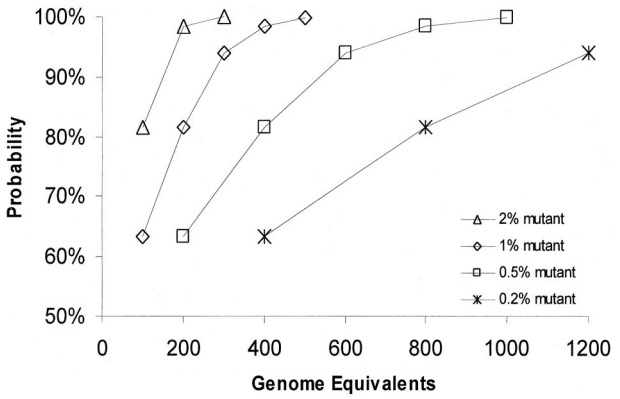

Figure 1. Probability of detecting rare mutant molecules. The theoretical probability of having at least a single mutant molecule present in a PCR reaction is shown as a function of the total input hDNA [expressed as genome equivalents (GE) per PCR] and percent mutant. The probability of detecting single events was calculated using Poisson statistics.

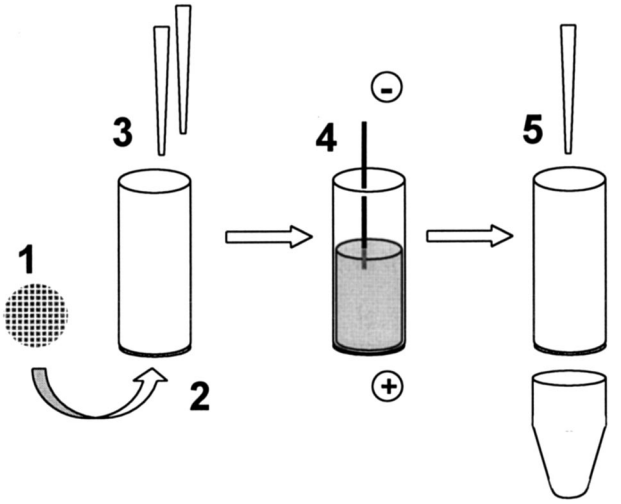

Figure 2. Schematic for the acrylamide gel sequence-specific capture method. The following steps are used to purify human DNA from homogenized stool samples: acrylamide-gel capture layers are prepared by copolymerizing Acrydite-functionalized oligos (20 μmol/L) with Bis-acrylamide in contact with a support mesh; disks of the capture membrane are sealed to the bottom of capture tubes; denatured samples are then added the tubes, followed by electrophoresis buffer; electrodes are positioned above and below the capture membrane to drive the target DNA into the capture layer (and all non-target sequences and components through the capture membrane); the sample solution is then removed, the membrane washed, and then the target eluted into a sterile container. For simplicity the schematic shows a single well. The studies described here were run using 48 wells in parallel, and an array of 48 matching electrodes.

patients and from colonoscopy-negative subjects, and analyzed using the multi-target assay described above. In those studies the DNA was purified using a modified sequence-specific capture method and streptavidin magnetic beads.[3,25] The results from these studies are summarized in Table 1, yielding an aggregate sensitivity of 67% (95% CI: 60.3 to 73.9%) and specificity of 97% (95% CI 92.9 to 99.2%). These calculations come from analysis of a total of 196 stools from confirmed cancer patients, and an additional 141 stools from colonoscopy-negative subjects. To assess the relative performance of DNA purification methods, and the effect of increased recovery of target DNA on assay performance, samples were drawn from archives of these previous studies (samples stored at −80°C). Not all samples were available for repeat analysis (only 86 of the 196 cancer samples had sufficient quantities remaining for this study). However, for samples chosen, separate aliquots were re-purified using the bead-capture method and the newer acrylamide gel-capture method to directly compare results of purification methodology.

The motivation for developing a methodology to maximize the recovery of human DNA from stool (and other bodily fluids) is driven by the reduced probability of detecting rare mutant sequences in heterogenous mixtures. The bead-capture method yields sufficient quantity and purity of DNA for successful amplification of >99% of the PCR reactions analyzed in the studies cited here. However, whereas only a relatively small number of purified copies may be necessary for successful amplification, the total copies required for adequate representation of a mutant sequence, which represents a small percentage of the total human DNA content, must be higher. DNA from tumor cells may be present as only about 1% of the total human DNA fragments present in the overall stool sample since most cells sloughed into the lumen are apoptotic normal cells. Recovery of total human DNA from stool samples, and mutant percentages can both vary in clinical applications. The probability of adequately representing mutant molecules in PCR reactions can be modeled as a function of total input human DNA for different mutant percentages (Figure 1). To establish a recovery goal we assume that mutant sequences are present as 1% of the total human DNA, nominally. Therefore our goal was to maximize the number of samples that yield at least 500 GE/PCR of human DNA.

Increased Recovery of Human DNA from Fecal Samples

To achieve increased genome equivalent yields (ie, >500 GEs) from all samples, we developed a novel method based on capture probes immobilized in an acrylamide gel layer. Capture probes are first end-labeled with acrylamide groups during sysnthesis[23] so that they can be copolymerized during preparation of an acrylamide gel. This approach has been previously reported in diagnostics applications,[24] but was modified here for sample preparation. The same capture-probe sequences that had been used in the magnetic bead purification method were modified to replace biotin groups with Acrydite groups. Acrylamide gel layers were then prepared and sealed to the bottom of receptacles that could hold up to 10 ml of stool sample homogenized in buffer (see Materials and Methods). A schematic of the gel-capture format is shown in Figure 2. Samples are electrophoresed through the gel-capture membrane to hybridize targeted sequences allowing non-target sequences and other components to pass through. The capture layers were then washed and the DNA eluted.

To test the impact of the purification method on recovery of DNA from stool, and further to test the effect of recovery on assay performance, 186 stool samples were purified and analyzed using both the magnetic bead-

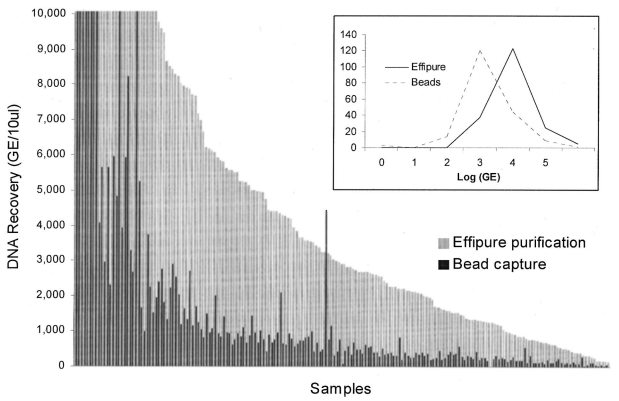

Figure 3. Evaluation of human DNA (*h*DNA) recovery from fecal samples using two purification methodologies. The median recovery of *h*DNA with an acrylamide gel sequence-specific purification method is 2880 GE/10 μl (a 5.4-fold increase over the bead-capture method; $P < 0.0001$). Overall 91% of samples meet the 500 GE/10 μl recovery target. Each **bar** represents a single sample. Results are sorted (high to low) for recovery using the gel-capture method (with the corresponding recovery value with bead-capture next to each sample). **Inset**, data are re-plotted as a logarithmic-scale histogram.

capture and the acrylamide gel-capture methods. The samples consisted of 100 stools from colonoscopy-negative patients, and 86 from confirmed cancer patients. Recovery was quantified for all samples using a sequence-specific real-time PCR method. Overall, the median recovery was 2880 GEs (per 10 μl) for the acrylamide-gel method, whereas for bead captures, the median recovery was 534 GE/10 μl. Overall, this represents an increase of 5.4-fold ($P < 0.001$ by a paired *t*-test) (Figure 3, shows the results sorted from high to low). It is also apparent that there is a broad range of inter-sample variability that is expected in population studies. With the acrylamide-gel method, recovery of human DNA from stool was >500 GE/10 μl for 91% of the samples. Additionally, 97% (86 of 89) of samples from cancer patients yield >500 GE while 86% (86 of 100) of samples from colonoscopy-negative patients yield >500 GE. This suggests that sufficient *h*DNA can be recovered from fecal samples using the acrylamide-gel method for maximizing representation and detection of rare mutant sequences when they exist. With the bead-capture method only 50% of the samples yielded a minimum of 500 GE/10 μl, and as a result it is expected that some rare mutants might not be detected by the multi-target assay due to insufficient representation in the PCR reactions.

Impact of Increased DNA Recovery on Detection of Mutations

All samples purified with the acrylamide-gel method were subjected to analysis with the multi-target assay. Because high false-positive rates (low specificity) are highly undesirable in a population screening test, we checked to see if the specificity of the multi-target assay had been compromised by an increase in DNA yield from the acylamide-gel purification. We first analyzed results for DNA from the colonoscopy-negative ("normal") patients. One sample was found to have a positive point mutation (k-ras; k12p2) and three normal samples were positive for the DIA marker, for a total calculated specificity of 96% (95% CI: 91.5 to 99.4%), which is similar to previous studies using bead captures (see Table 1). Analysis of the same samples purified by the bead-capture method showed three samples positive by the mutation panel. None of these were the same as the single false positive with the acrylamide gel-purified samples, although all three were also positive for *K-ras* (two for K12p2, and one for K13p2). Analysis of the same samples by the DIA portion of the multi-target assay revealed three positives, none of which overlapped with the mutation-panel markers. Therefore the combined clinical specificity calculated for bead capture was 94%, which is similar to the results for the matched samples purified with the acrylamide-gel method, as well as historical data (see Table 1).

To determine the impact of increased human DNA recovery on assay sensitivity, we analyzed the 86 cancer samples re-purified using both methods. Results are summarized in Table 2. All of the samples (100%) that tested positive after purification with the magnetic-bead method, also tested positive (with the same markers) using the acrylamide-gel method. However, additional cancer samples were also found to be assay positive with the acrylamide-gel capture. An additional six cancer samples were detected by the mutation panel portion of the multi-target assay. In total 12 additional point mutation markers were detected, although six of these were found in samples with multiple positive markers, and therefore did not add to the clinical sensitivity of detection. Samples are all from archives (stored at −80°C) and it is possible to compare results from this analysis to the initial test results. Originally, 36 cancer samples were found to be positive for the mutation panel portion of the assay, and all of these were again confirmed to be positive with bead capture, indicating good reproducibility of the assay on repeat testing the same samples. The DIA portion of the assay reveals 19 additional cancer samples that were detected after acrylamide-gel purification, compared to bead capture. Similar to the mutation panel portion of the assay, we found 100% concordance between samples that had been found to be positive originally, and those that were positive on repeat testing.

To assess overall improvement in detection of cancers the overlap of the mutation panel and DIA portions of the multi-target assay is taken into account. With some cancers, point mutations are detected in addition to detection of long DNA (note that for this reason we cannot simply add the number of samples found positive by the mutation panel and DIA). We find that three of the six new mutation markers overlap with samples that are positive by the DIA marker. Likewise we find that 8 of the 19 added DIA positive samples overlap with the mutation panel portion of the assay. However, taken together, 14 additional cancer samples are detected when DNA is purified with the acrylamide-gel method compared to the bead-capture method. By direct comparison this suggests an assay sensitivity increase from 53% (95% CI = 42 to 64%) to 70% (95% CI = 59 to 79%). These findings are summarized in Table 3. The assay sensitivity increase was found to be statistically significant by McNemar's test ($P = 0.0005$).

Table 2. Results of the Multi-Target Assay Analysis for All Cancer Samples

Sample ID	Original study	Clinical status	Mutation panel assay		DNA integrity assay	
			Bead capture	Gel capture	Bead capture	Gel capture
PV31	A	CIS/HGD	POS (10)	POS (10)	Neg	Neg
PV151	A	CIS/HGD	neg	neg	Neg	Neg
PV6	A	Dukes A	neg	neg	POS	POS
PV7	A	Dukes A	neg	neg	POS	POS
PV12	A	Dukes A	POS (14)	POS (14)	POS	POS
PV13	A	Dukes A	neg	neg	POS	POS
PV15	A	Dukes A	POS (17)	POS (17,12)	Neg	Neg
PV18	A	Dukes A	neg	neg	POS	POS
PV19	A	Dukes A	POS (3,14)	POS (2,3,14,20)	Neg	Neg
PV152	A	Dukes A	neg	neg	Neg	Neg
PV153	A	Dukes A	neg	neg	Neg	POS
PV154	A	Dukes A	neg	neg	Neg	POS
PV21	A	Dukes B	POS (22)	POS (22)	POS	POS
PV24	A	Dukes B	neg	POS (7)	POS	POS
PV28	A	Dukes B	POS (14)	POS (14)	Neg	POS
PV29	A	Dukes B	POS (20)	POS (20)	POS	POS
PV155	A	Dukes B	neg	POS (1)	POS	POS
PV156	A	Dukes B	neg	neg	Neg	POS
PV157	A	Dukes B	neg	neg	Neg	POS
PV158	A	Dukes B	neg	neg	Neg	Neg
PV159	A	Dukes C	neg	neg	Neg	Neg
PV160	A	Dukes C	neg	neg	Neg	Neg
PV161	A	Dukes C	neg	neg	Neg	Neg
PV162	A	Dukes C	neg	POS (12)	Neg	Neg
PV163	A	Dukes C	neg	neg	Neg	Neg
PV164	A	Dukes C	neg	neg	Neg	POS
PV33	A	Dukes D	POS (10)	POS (10)	Neg	Neg
PV165	A	Dukes D	neg	neg	Neg	Neg
PV167	B	CIS	neg	neg	Neg	POS
PV48	B	CIS	neg	neg	POS	POS
PV53	B	Dukes A	POS (2)	POS (2)	Neg	Neg
PV34	B	Dukes A	POS (14)	POS (14)	Neg	POS
PV171	B	Dukes A	neg	neg	Neg	Neg
PV36	B	Dukes A	POS (14)	POS (14)	Neg	Neg
PV58	B	Dukes B	POS (22)	POS (22)	Neg	POS
PV60	B	Dukes B	POS (22)	POS (22)	Neg	Neg
PV38	B	Dukes C	POS (13)	POS (13)	Neg	POS
PV39	B	Dukes C	POS (13)	POS (13)	Neg	Neg
PV40	B	Dukes C	POS (3)	POS (3)	Neg	Neg
PV42	B	Dukes C	POS (2)	POS (2)	POS	POS
PV46	B	Dukes C	POS (7)	POS (7)	Neg	Neg
PV172	B	Dukes C	neg	neg	Neg	Neg
PV47	B	Dukes C	POS (7)	POS (7)	Neg	Neg
PV54	B	Dukes C	neg	neg	POS	POS
PV168	B	Dukes C	neg	neg	Neg	Neg
PV169	B	Dukes D	neg	neg	Neg	Neg
PV170	B	Dukes D	neg	neg	Neg	Neg
PV166	B	Dukes D	neg	POS (7)	Neg	Neg
PV67	C	Dukes A	POS (15)	POS (1,15)	Neg	Neg
PV68	C	Dukes A	POS (22)	POS (22)	Neg	Neg
PV80	C	Dukes A	neg	POS (3)	POS	POS
PV98	C	Dukes A	POS (3,6)	POS (3,6)	Neg	POS
PV102	C	Dukes A	POS (2)	POS (2)	POS	POS
PV174	C	Dukes A	neg	neg	Neg	Neg
PV178	C	Dukes A	neg	neg	Neg	Neg
PV180	C	Dukes A	neg	neg	Neg	Neg
PV181	C	Dukes A	neg	neg	Neg	Neg
PV182	C	Dukes A	neg	neg	Neg	Neg
PV184	C	Dukes A	neg	neg	Neg	Neg
PV185	C	Dukes A	neg	neg	Neg	POS
PV188	C	Dukes A	neg	neg	Neg	Neg
PV65	C	Dukes B	POS (2,4)	POS (2,4)	Neg	Neg
PV69	C	Dukes B	POS (4)	POS (4)	POS	POS
PV71	C	Dukes B	POS (22)	POS (22)	Neg	Neg
PV101	C	Dukes B	POS (4,14)	POS (4,14)	POS	POS
PV175	C	Dukes B	neg	neg	Neg	POS
PV177	C	Dukes B	neg	neg	Neg	POS
PV190	C	Dukes B	neg	neg	Neg	POS

Table 2. Continued

Sample ID	Original study	Clinical status	Mutation panel assay		DNA integrity assay	
			Bead capture	Gel capture	Bead capture	Gel capture
PV191	C	Dukes B	neg	neg	Neg	Neg
PV70	C	Dukes C	POS (2)	POS (2)	POS	POS
PV75	C	Dukes C	neg	neg	POS	POS
PV55	C	Dukes C	POS (3,17)	POS (3,17)	Neg	POS
PV77	C	Dukes C	POS (14)	POS (14)	POS	POS
PV86	C	Dukes C	POS (2,22)	POS (2,22)	Neg	Neg
PV87	C	Dukes C	POS (2)	POS (2)	Neg	POS
PV88	C	Dukes C	POS (2,20)	POS (2,20)	POS	POS
PV93	C	Dukes C	POS (9)	POS (9,16)	Neg	Neg
PV103	C	Dukes C	POS (17)	POS (17)	POS	POS
PV173	C	Dukes C	neg	neg	Neg	Neg
PV176	C	Dukes C	neg	neg	Neg	Neg
PV183	C	Dukes C	neg	POS (19)	Neg	Neg
PV186	C	Dukes C	neg	neg	Neg	Neg
PV187	C	Dukes C	neg	neg	Neg	Neg
PV189	C	Dukes C	neg	neg	Neg	Neg
PV192	C	Dukes C	neg	neg	Neg	POS
PV76	C	Dukes D	POS (21)	POS (21,13)	Neg	POS

Marker designations are as follows: (1) k12p1 (k-ras); (2) k12p2 (k-ras); (3) k13p2 (k-ras); (4) 1309 (APC); (5) 1306 (APC); (6) 1312 (APC); (7) 1367 (APC); (8) 1378 (APC); (9) 1379 (APC); (10) 1450 (APC); (11) 1465 (APC); (12) 876 (APC); (13) 1554 (APC); (14) 175p2 (p53); (15) 245p1 (p53); (16) 245p2 (p53); (17) 248p1 (p53); (18) 248p2 (p53); (19) 273p1 (p53); (20) 273p2 (p53); (21) 282p1 (p53); (22) BAT-26. The study designations are as follows: A, Tagore;[11] B, Brand;[10] C, Syngal.[12] Clinical status refers to cancer stage; CIS, carcinoma in situ; HGD, high-grade dysplasia.

Discussion

Many investigators have noted the paucity of DNA contributed by tumor cells sloughed into the colonic lumen compared with DNA contributed by sloughed normal, dead cells of the colonic epithelium. [3,26,27,28] DNA from tumor cells would be expected to represent a very small percentage of the total human DNA that is present in stool specimens, as the majority of cells would be expected to be normal epithelium. A further assumption of detection is that cancer and normal cells are equally likely to be sloughed and that their DNA is equally likely to remain intact in stool, a destructive and heterogeneous material. Any cancer screening test that depends upon analyzing genes or genome status in tumor cells found in stool is therefore limited by a requirement for sufficient representation of DNA from tumor cells among the total human DNA in a stool sample such that there is high probability of detecting the tumor DNA. For instance, when purifying DNA from fecal samples it is not only necessary to stabilize DNA from degradation, and mitigate inhibition of PCR, it is also necessary to ensure that recovery of DNA is maximized to detect rare mutant populations, when they exist. A bead-capture method that had been developed for DNA retrieval in earlier studies was adequate but not optimal. By referring to a statistical model (based on a subjectively selected nominal mutant population of 1%), requiring a minimum recovery of 500 GE per PCR for increased probability of detection, we found insufficient human DNA was recovered from 50% of stool samples using the bead-capture method. This led us to develop methods for increased human DNA recovery from stool, and to test the impact of increased recovery on our multi-target assay performance.

The acrylamide gel-capture method provided greater recovery of human DNA, compared to a previously developed bead-capture method when 186 stool samples were processed in parallel. The increase in DNA-recovery varied from sample to sample, and was as high as 30-fold. The average increase was 5.4-fold ($P < 0.0001$, by the paired *t*-test). The ability to capture more genome equivalents of human DNA with the acrylamide-gel method can be explained by several features of the methodology: the matrix of conjugated acrylamide has a high capacity for the human-specific capture probes such that the capture sites are not saturated by target DNA from stool; the sample is applied to the capture probes by electrophoresis, thus driving the target DNA to the capture probes in an efficient manner (bead captures by comparison rely on mass transfer via diffusion); the acrylamide matrix minimizes non-specific adsorption of non-

Table 3. Summary of Multi-Target Assay Sensitivity Analysis

	Mutation panel		DIA		Combined assay	
	Bead capture	Gel capture	Bead capture	Gel capture	Bead capture	Gel capture
Pos. samples	36	42	21	40	46	60
Total samples	86	86	86	86	86	86
Sensitivity	42%	49%	24%	47%	53%	70%
(95% CI)	(31–53%)	(38–60%)	(16–35%)	(36–58%)	(42–64%)	(59–79%)

target biomolecules, which minimizes inhibition of PCR reactions downstream. Due to these features, it is possible to readily scale-up the volume of patient sample in each capture experiment, for greater DNA yield [attempts to recover more human DNA by scaling the magnetic-bead method were unsuccessful due to PCR inhibition for an unacceptably high percentage of samples (data not shown)].

On purification and analysis of cancer samples, an increased number were detected when DNA recovery was increased, using the acrylamide-gel method. Our hypothesis is that this is due to minimized sampling error in PCR reactions when the mutant population is present in a small percentage of the total human DNA (as with fecal samples). There were three added cancer samples that were uniquely positive by the mutation panel portion of the assay. Using bead capture they were found to have recovery scores of 300, 210, and 190 GE/10 μl, respectively. Clearly all three fall below the DNA recovery target (500 GE/10 μl). When purified with the gel-capture method, these same samples gave scores of 1210, 1180, and 1630 GE/10 μl, respectively.

The majority of the increase in sensitivity was contributed by the DIA component of the multi-target assay. This result was somewhat unexpected, and the precise mechanism by which increased recovery leads to increased sensitivity is not clear at this point although we hypothesize that increased recovery of long fragments is sufficient for increased sensitivity due to the increase in template amount that leads to increased PCR efficiency.

High specificity is requisite for cost-effective screening. Four false positives were identified in this study, one due to a k-ras mutation and three to positive DIA readings. The new technique reported here for purification of human DNA from stool results in increased recovery and appears to improve the sensitivity in detecting colorectal cancer while maintaining high specificity. This should improve the robustness of the multi-target DNA assay described here as a tool for colorectal cancer screening. We also believe that other molecular techniques that rely on rare mutant detection would benefit from this recovery and purification approach.

As expected from the choice of markers, cancers at all tumor stages (Dukes A-D) were readily detected in fecal DNA analysis. No statistically significant correlation of sensitivity or specificity was observed with disease stage using either the bead-capture or the gel-capture methods. Likewise, no correlation of DNA recovery was observed with disease stage. However, we observed that there is clearly a correlation of sensitivity with recovery and that increased recovery of human DNA using the gel-capture method led to increases in sensitivity for all tumor stages. More importantly, early-stage tumors, associated with greater survival odds, are detected as readily as late-stage tumors.

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